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13. ABSTRACT (Maximum 200 Words)  In order to identify and characterize additional novel or unexpected proto-oncogenes that, in addition to fibroblast growth factors (Fgfs) cooperate with <i>Wnt1</i> in murine mammary tumorigenesis, we have generated MMTV-infected <i>Wnt10b/DN-Fgfr2</i> bitransgenic mice. While the Wnt oncogenic signal is constitutively overexpressed in their mammary gland, cooperative oncogenic Fgf signals should be abolished by the expression of a dominant-negative FGF receptor ( <i>DN-Fgfr2</i> ). Only those cells carrying MMTV-insertionally activated cellular proto-oncogenes, other than Wnts and Fgfs, should have a growth advantage in the bitransgenic mammary gland. The clonal expansion of these cells leads to mammary tumorigenesis. As proposed, we have generated a cohort of 25 MMTV-infected <i>Wnt10b/DN-Fgfr2</i> bitransgenic females. As controls, we have also generated an uninfected bitransgenic cohort, as well as MMTV-infected/uninfected <i>Wnt10b</i> and <i>DN-Fgfr2</i> female groups. To date, several mammary adenocarcinomas have appeared in the MMTV-infected bitransgenic animals. At least three tumors carry newly integrated MMTV proviruses in loci other than <i>Wnt1</i> . Expression of the most common <i>Fgf</i> targets of MMTV insertional activation ( <i>Fgf3</i> , <i>Fgf4</i> , <i>Fgf8</i> ) has not been detected in the analyzed tumors. These, therefore, appear as potential candidates harboring other insertionally activated genes. We are currently trying to clone and identify these genes, and are also screening for additional candidate tumors			
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## INTRODUCTION

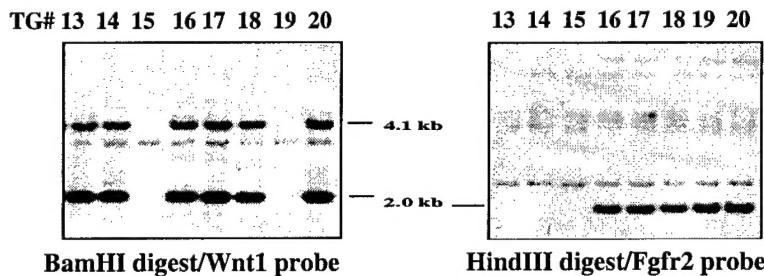
Mouse mammary tumor virus (MMTV) **insertional mutagenesis in transgenic mice** is a very useful approach to identify novel or unexpected proto-oncogenes implicated the development of mammary tumors. The mouse mammary tumor virus (MMTV) is biological carcinogen that induces murine mammary tumorigenesis by the mechanism of insertional mutagenesis (1-4). MMTV proviral integration in the vicinity of cellular proto-oncogenes may result in activation of their expression. This event confers a selective growth advantage to the mutated mammary epithelial cells and facilitates their clonal outgrowth, eventually leading to tumor formation. In tumor DNAs containing newly integrated proviruses, MMTV is physically linked to adjacent insertionally activated proto-oncogenes, thus it can be used as a molecular tag that facilitates the cloning and identification of the activated gene. Analysis of the MMTV integration loci in mammary tumors from MMTV-infected *Wnt1* or *Fgf3*-transgenics has revealed preferential activation and expression of either *Fgf* genes (*Fgf3*, *Fgf4*, *Fgf8*) in *Wnt1* transgenics or activation of *Wnt* genes (*Wnt1*, *Wnt10b*) in *Fgf3* transgenics (6-11). This fact, together with the decreased tumor latencies observed in *Wnt1/Fgf3* bitransgenics, demonstrate that activation of *Wnt* and *Fgf* genes, and strong oncogenic cooperation between both growth factor families, are crucial events involved the molecular basis of multistep mammary tumorigenesis (12). Nonetheless, little is known to date about additional genes that collaborate in this malignant process.

We propose to use MMTV-insertional mutagenesis in a *Wnt10b/DNFgfr2* bitransgenic mouse model, to identify and characterize novel or unexpected proto-oncogenes that, in addition to *Fgfs*, cooperate with *Wnts* in multistep mammary tumorigenesis. In MMTV-infected *Wnt10b/DNFgfr2* bitransgenic mice, the *Wnt* oncogenic signal will be constitutively overexpressed in the mammary gland (13,14). However, cooperative oncogenic *Fgf* signals will be abolished by the expression of a truncated form of the fibroblast growth factor receptor 2 (*DNFgfr2*). The modified receptor functions in a dominant-negative fashion, thus blocking *Fgf* signaling mediated by endogenous *Fgfrs* (15-19). We expect that only those cells carrying insertionally activated cellular proto-oncogenes, other than *Wnts* and *Fgfs*, have a growth advantage in the bitransgenic mammary gland. The clonal outgrowth of these specific cell populations can develop into mammary tumors. These can be then analyzed to isolate and identify the activated proto-oncogenes.

**REPORT BODY****Task 1. Generate MMTV-infected *Wnt10b/DN-Fgfr2* bitransgenic mice (months 1-5)**

- A. Establish matings between *Wnt10b* and *DN-Fgfr2* single transgenics**
- B. Southern blot analysis of positive transgenic offspring**

*Wnt10b* and *DNFgfr2* transgenic lines were previously generated in my mentor's laboratory. These mice were bred and their offspring screened by Southern blot analysis to identify the bitransgenic animals as follows. Blots from *HindIII* or *BamHI* genomic DNA digests were probed with *DNFgfr2* cDNA or with *Wnt10b* cDNA respectively to detect the presence of each transgene (Fig.1). Offspring positive for both transgenes were further bred to establish the *Wnt10b/DNFgfr2* bitransgenic mouse lines. A total of 320 mice have been generated and screened to date.



**Figure 1. Southern Blot Screening of *Wnt10b/DNFgfr2* bitransgenic mice. Left panel: *Wnt10b* transgenic mice display 2.5 and 4.5 kb bands. Right panel: *DNFgfr2* transgenic mice display a 2.0 kb band.**

**C. Grow MMTV<sup>supF</sup> XC rat cells****D. Infect, via intra-peritoneal (IP) injection, *Wnt10b/DNFgfr2* bitransgenic females and non-transgenic littermates**

Stably transfected rat XC sarcoma cell clones, that produce different MMTV variants, were previously generated in my mentor's lab. We have characterized the levels of MMTV expression in these clones by northern blot analysis of their total RNA (data not shown). The expression level of MMTV in the EH-*supF*9 clone was considered suitable for our experiment, and this clone was further expanded and used for MMTV infection (*via* IP injection) of our mice (20).

Six mouse cohorts (n=25 mice each) have been established for our insertional mutagenesis studies. A *Wnt10b/DNFgfr2* bitransgenic mouse cohort, and control female *Wnt10b* or *DNFgfr2* single transgenic littermate cohorts (3-4 weeks old) have been infected with MMTV

or DNFgfr2 single transgenic littermate cohorts (3-4 weeks old) have been infected with MMTV EH-*supF9*. Similar cohorts of uninfected animals are also used as controls. All animals are actively bred in order to stimulate the propagation of MMTV infection in the proliferating glands. The animals are examined weekly for the development of mammary tumors.

**Task 2. Obtain mammary tumor tissue and nucleic acids from MMTV-infected *Wnt10b/DNFgfr2* bitransgenic mice (months 6-11)**

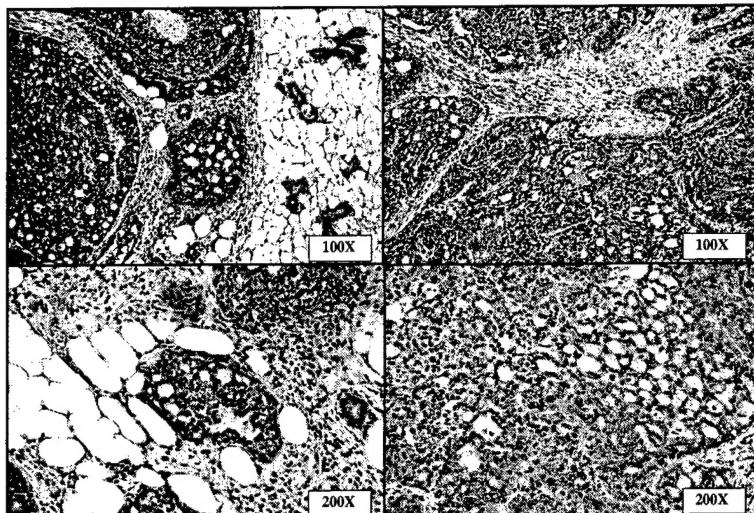
- A. Conduct weekly physical examination of the mice to detect the development of mammary tumors**
- B. Surgically resect, under anesthesia, the mammary tumors**
- C. Process tumor samples for histopathology and tumor DNA and RNA analysis**

During a six month-long period following infection with MMTV-EH *supF9*, only two bitransgenic animals developed mammary tumors. This represents a lower incidence than initially expected. Unrelated experiments done by others in my mentor's laboratory suggest that our *Wnt10b* mice may be losing transgene expression overtime. This fact may be contributing to the low tumor incidence and prolonged latency that we have observed in our bitransgenic animals. We have also considered the possibility that our mice may not be efficiently infected with the MMTV-EH *supF9* retrovirus, due perhaps to some unknown incompatibility between the viral strains and the infection susceptibility in the mouse strain used in our experiments. In order to circumvent this latter potential problem, our bitransgenic mouse cohort was re-infected with a *wild-type* C3H MMTV strain, produced by the Mm5MT mouse cell line, by injecting these cells into mice as done with the EH-*supF9* cells. This wild-type virus has been reported to strongly infect and induce mammary tumor formation in mouse strains similar to ours.

Approximately one month after the new infection, 50% of the females developed very large tumors, always located around the injection area; some of these animals also developed massive intra-abdominal tumors. The very short latency, and the constant location of the new tumors, suggested that the producer Mm5MT cells might have not been rejected by the infected animals and have themselves produced the tumors. Southern blot analysis of the tumor DNAs has confirmed this possibility. We now feel that those tumors that quickly arose after the second round of infection are not good candidates for analysis in our retroviral insertional mutagenesis study.

From the 50% of bitransgenic females that remained tumor-free after C3H-infection, five animals have developed a total of seven independent mammary adenocarcinomas, which have been confirmed as such by histopathology (Fig. 2). Because these tumors appeared with a longer latency (2-5 months post-injection) and are located in distant locations from point of injection,

we feel that these tumors may be indeed arising as the result of C3H infection and insertional mutagenesis of mammary epithelial cells.



**Figure 2. Histopathology of *Wnt10b/DNFgfr2* mammary tumors.** The figure shows abnormal proliferation of ductal, lobular, and connective structures that invade the normal mammary adipose parenchyme (top panels: 100X magnification, bottom panels: 200X magnification; Hematoxylin-Eosin stain)

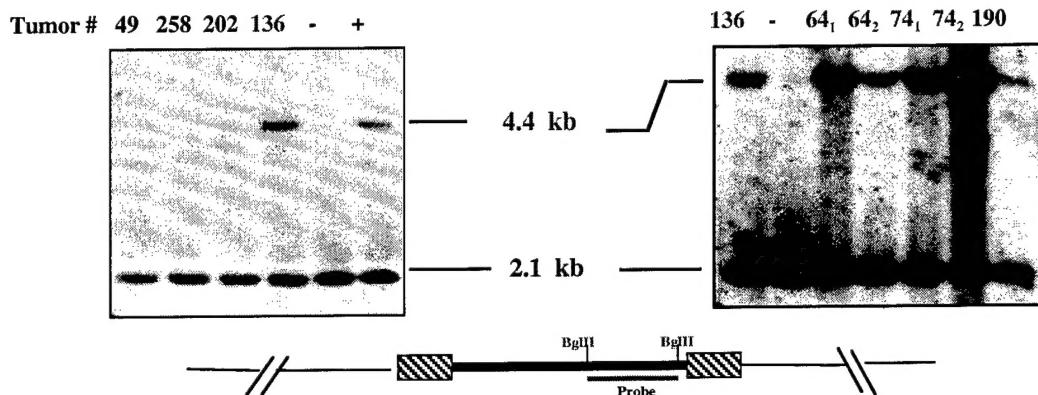
To date, these seven tumors, together with those that arose before the second MMTV infection, have been analyzed as follows.

**Task 3. Identify MMTV-proviral-cellular DNA junction fragments and clone the cellular sequences contained in them (months 12-19)**

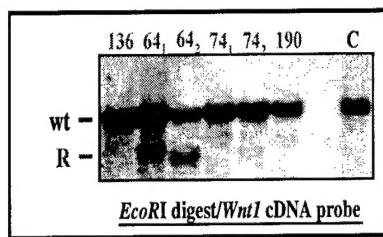
- A. Screen, by Southern blot, the tumor DNAs for new MMTV<sup>supF</sup> integrations**
- B. Clone the junction fragments into appropriate  $\lambda$ -phage vector**
- C. Package and titer phages and infect appropriate *E.Coli* hosts**
- D. Select positive clones and subclone the cellular sequences into plasmid vectors**

The presence of newly integrated proviruses was detected has been detected by Southern blot in six of the tumors (Fig. 3). This analysis also provided solid evidence that the tumors are derived from mouse tissue and not Mm5MT cells. Of the original nine tumors, two of them have appeared in the last 2 months, and remain to be screened for proviral integrations. Southern blot analysis also revealed that two of these tumors display rearrangements in the *Wnt1* locus, suggesting that *Wnt1* activation by MMTV may be the mechanism involved in their generation, and will thus not be analyzed further (Fig. 4). Currently we are performing Northern blot analysis of the expression of *Wnt1* and *Fgf3* (two oncogenes that are commonly activated by MMTV in mammary tumors). Some of the tumors analyzed thus far display a lack of activation of either gene (Fig. 5). We will also test for expression of other common insertionally activated genes (*Wnt3*, *Fgf4*, *Fgf8*). After our screening is completed we will proceed to clone viral-

cellular junction fragments from those tumors not displaying abnormal activation of the tested *Wnt* and *Fgf* genes (as described in the Materials and Methods section of the original proposal).

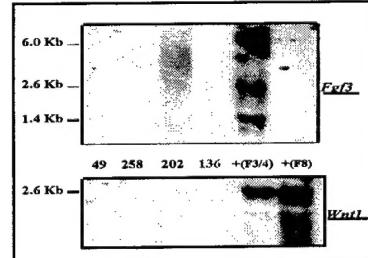


**Figure 3. Newly integrated proviruses in *Wnt10b/DNFgfr2* mammary tumors.** Southern blot analysis was performed on a *Bgl*II digest of tumor DNA (10  $\mu$ g) to release an internal MMTV specific fragment (see sketch). The 4.4 kb band indicates the presence of the newly integrated proviruses. The 2.1 kb band corresponds to endogenous MMTV present normally in these mice. (-): Uninfected mouse tail DNA. (+): *Wnt1* tumor DNA with a known new MMTV provirus integration.



**Figure 4. Southern blot analysis of *Wnt1* locus rearrangements in *Wnt10b/DNFgfr2* tumors.**

Six independent mammary tumor DNAs were screened for the presence of rearranged (R) bands in the *Wnt1* gene locus. Tumors # 136, 741, 742, and 190 display a wild-type (wt) locus band pattern. C: Control *Wnt10b/DNFgfr2* tail DNA



**Figure 5. Representative northern blot analysis of *Wnt1* and *Fgf3* expression in *Wnt10b/DNFgfr2* mammary tumors.** Total tumor RNAs (20  $\mu$ g) were used. Blots were probed with *Fgf3* (top panel) or *Wnt1* (bottom panel) cDNA. The expected gene transcript size (kb) is indicated on the left side. Tumor numbers are indicated between the panels. +(F3/4): Control tumor RNA with known activation of *Fgf3* and *Fgf4* expression. +(F8): Control tumor RNA with known *Fgf8* activation

**KEY RESEARCH ACCOMPLISHMENTS**

1. Generation of *Wnt10b/DNFgfr2* mice
2. MMTV-infection of *Wnt10b/DNFgfr2*, *Wnt10b*, and *DNFgfr2* females
3. Induction of mammary adenocarcinomas in *Wnt10b/DNFgfr2* females
4. Confirmation of the existence of new MMTV proviral integrations and the clonal origin of the *Wnt10b/DNFgfr2* mammary adenocarcinomas.
5. Partial characterization of expression levels for various *Wnt* and *Fgf* oncogenes in the *Wnt10b/DNFgfr2* mammary tumors

**REPORTABLE OUTCOMES**

1. Generation of MMTV-infected *Wnt10b/DNFgfr2* bitransgenic mice
2. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. December 28, 2000. Avances en Biología Molecular por Jóvenes Investigadores en el Extranjero. Centro Nacional de Biotecnología, Madrid, Spain.
3. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. November 18, 2000. University of Southern California, Los Angeles, California.
4. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. February 16, 2001. Childrens Hospital Los Angeles, Los Angeles, California.

**CONCLUSIONS**

During the past twelve months, we have accomplished the generation of MMTV-infected *Wnt10b/DNFgfr2*. The oncogenic cooperation between member of the *Wnt* and *Fgf* gene families is a crucial and well known molecular event implicated in the development of mammary tumors in mice. The use of MMTV-insertional mutagenesis in a *Wnt/DNFgfr* model is therefore a logical step when trying to elucidate what additional genetic events are involved in. Our MMTV-infected *Wnt10b/DNFgfr2* mouse model constitutes the first reported contribution to taking to take the multistep mammary tumorigenesis studies one step beyond. To date, several mammary tumors have appeared in the MMTV-infected bitransgenics. Molecular analysis of

these tumors that at least six of them may be potential candidates to harbor insertional activations of novel or unexpected oncogenes other than Wnts or Fgfs. The identification and cloning of such genes is our next immediate goal.

We and others have improved several experimental strategies (see Materials and Methods of original proposal) that will facilitate the cloning of such genes. In addition, the recent and upcoming advances made in the completion of the mouse and human genome projects will certainly be used to expedite the identification of the candidate genes. We therefore feel that our immediate goal will be successfully achieved in the coming year.

**Recommended changes to the original proposal.** In order to circumvent the problems encountered in the generation of large numbers of mammary tumors in the MMTV-infected *Wnt10b/DNFgfr2* females, we have undertaken the task of developing MMTV-infected *Wnt1/DNFgfr2* and *Wnt1/Fgf3* bitransgenic animals. With this purpose we intend to use the following founders: *Wnt1* male (B6SJL[*Wnt1*]HeV, from the Jackson Laboratory), *DNFgfr2* females (C57B6SJL/J, generated previously in our lab), and *Fgf3* females (FVB, kindly provided by Dr. Philip Leder's laboratory).

Although the use of these groups of mice represents a change to our proposed project, the same working hypothesis and rationale, as originally postulated for our MMTV-infected *Wnt10b/DNFgfr2* bitransgenics, apply for the *Wnt1/DNFgfr2* experimental group. In a similar fashion, for the MMTV-infected *Wnt1/Fgf3* bitransgenics, we expect that constitutive overexpression of *Wnt1* and *Fgf3* oncogenic signals will lead to the genesis of clonal mammary tumors displaying the insertional activation of oncogenes that cooperate with both *Wnts* and *Fgfs* in multistep mammary tumorigenesis. In this way, while we still intend to continue with the analysis of the seven potential *Wnt10b/DNFgfr2* tumor candidates, we will ensure that we have access to potential candidate tumors in which we can conduct our insertional mutagenesis and oncogenic cooperation studies. Moreover, it is also possible that the variation in the constitutive *Wnt* and *Fgf* overexpression pattern of each experimental group may lead to the identification of a wider range of novel or unexpected cooperating oncogenes.

With the purpose of avoiding any unforeseen immunogenic incompatibilities between the mouse strain, viral strain, and the cells lines used for injection, we suggest another variation in our experimental design. Instead of the proposed injection (via IP) of MMTV-producing cells, we intend to deliver multiple subcutaneous injections of *wild-type* MMTV (C3H) virus (approx.  $1.55 \times 10^{11}$  viral particles/injection site). It is generally accepted that, upon infection of the host, MMTV spreads to the mammary gland through the infection, expansion, and migration of infected B-lymphocytes throughout the body (21-23). We have confirmed that subcutaneous

MMTV injection induces appropriate regional lymph node reactivity and that MMTV proviral DNA can be detected in the lymph node DNA analyzed by PCR several days after injection (not shown). We therefore believe that the use of this modality of MMTV delivery will ensure successful mammary gland MMTV infection in the new bitransgenic injected cohorts.

At this moment we are ready to complete the generation of the new MMTV-infected bitransgenic female cohorts and to proceed with a similar experimental approach to that stated in our original proposal. Due to the reasons stated above, we are confident that the addition of the mentioned changes will result in an enhanced capability to generate large numbers of tumors, and that their analysis will lead to the identification of new oncogenes involved in the development of breast tumors.

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